

Partial Purification of Antimicrobial Compounds Isolated from Mycelia of Tropical *Lentinus cladopus* LC4

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Lentinus cladopus LC4 produced at least eight antimicrobial compounds (ACs) which are active against plant and human pathogens. Three ACs in its crude mycelial were extracted with methanol and partial purification was carried out with silicic acid column chromatography and by thin layer chromatography (PTLC). The antimicrobial activity was tested by paper disc method and antibiographic method. The chromatography purification eluted with dichloromethane containing 5% methanol gave one active fraction (FII). This fraction which was active against *X. campestris* pv. *glycines* and showing two inhibition zones against *Bacillus subtilis* on bioautographic plates with the R_f s 0.8 and 0.7. FI and FIII fractions eluted with dichloromethane containing 0 and 10% methanol performed one inhibition zone with R_f s 0.8 and 0.7 respectively. However, their activities were lower than that of FII fraction. The PLTC purification gave one separate fraction with R_f value of 0.73 and it was active against *X. campestris* pv. *glycines*. The compound of R_f 0.73 fraction should be further studied using TLC and HPLC to obtain the pure substance for molecule characterization.

Key words: tropical *Lentinus cladopus* LC4, antimicrobial compounds, partial purification

INTRODUCTION

Mushrooms have been applied throughout the world as both food and medicine for thousands of years. Two thousand years have passed since the first century Greek physician Dioscorides included the polypore *Fomitopsis officinalis*, as a treatment for tuberculosis, in his *De Materia Medica* published approximately 65 C.E. The potential of mushrooms (macrofungi) as sources of antibiotics was reported by Anchel, Hervey, Wilkins in 1941 (Sandven 2000). They tested the extracts of fruiting bodies and mycelia culture from over 200 species. Several compounds that inhibit the growth of a large spectrum of saprophytic and phytopathogenic fungi and bacteria were isolated from basidiomycetes (Anke 1989; Coletto *et al.* 1994; Anke 1995; Bianco & Striano 2000; Smania *et al.* 2001; Badalyan *et al.* 2008; Minato 2008). The study on polypores, such as several species of *Ganoderma*, *Trametes versicolor*, *T. Marianna*, *T. cingulata*, and *Laetiporus sulphureus* and gilled mushrooms, such as *P. ostreatus*, *Lentinus connatus*, and *Lentinula edodes* showed either the antibacterial, anti-candida, antiviral or cytotoxic activities (Hirasawa *et al.* 1999; Gerasimenya *et al.* 2002; Babitskaya *et al.* 2003; Rukachaisirikul *et al.* 2005; Turkoglu *et al.* 2006; Ofodile *et al.* 2008). Despite their potential and enormous diversity in tropical ecosystems, few studies aiming at the discovery of bioactive compounds from mushrooms were conducted in Indonesia.

A number of species of *Lentinus* are edible and supposedly therapeutic properties and traditionally are used by local peoples, several among them are *L. sajor-caju*, *L. squarrosulus*, *L. badius* (Pegler 1983), and *L. tuberregium* (Isikhuemhen *et al.* 2000). Several other antimicrobial compounds have already been discovered from *L. crinitus* (Abraham & Abate 1995), *L. degener* (Anchel *et al.* 1948), and *L. adhaerens* (Lauer *et al.* 1991). In addition several metabolites were produced by *Lentinus* as the fragrance compounds from *L. lepideus* (Hanssen & Abraham 1987), the antiaggregant compound from *L. adhaerens* (Lauer *et al.* 1991), and the antitumor substances from *L. lepideus* and *L. trabeum* (Jong & Gantt 1987).

On silica gel thin-layer chromatograms, the crude extracts of tropical *Lentinus* were separated into several bioautographic spots; for the filtrate extracts of *L. squarrosulus* 55A into three spots (R_f s 0.75, 0.50, and 0.17), the mycelial extracts of *L. sajor-caju* LSC8 into two spots (R_f s 0.77 and 0.54), the mycelial extract of *L. torulosus* LU3 into two spots (R_f s 0.77 and 0.48), the filtrate extracts of *L. cladopus* LC6 into one spot (R_f 0.76) but the mycelial extracts of this mushroom separated into two spots (R_f s 0.79 and 0.54), the filtrate and mycelial extracts of *L. cladopus* LC4 into three spots respectively (R_f s 0.75, 0.61, and 0.45 for the filtrate extract and R_f s 0.83, 0.73, and 0.60 for mycelial extract) (Sudirman 2005), while the fruiting body extract into two spots (R_f s 0.68 and 0.08) (Sudirman 2009, unpublished data).

This work is part of a screening program aiming to discover new bioactive metabolites from tropical mushrooms. We reported here the results of purification

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process of three antimicrobial compounds of *L. cladopus* LC4 by column chromatography and preparative thin layer chromatography.

MATERIALS AND METHODS

Fungal Isolate. *L. cladopus* LC4 isolate was obtained by mating of two compatible monokaryon mycelia come from spores which were isolated from a fruiting body of *L. cladopus* LC. Bacterial plant pathogens, *Xanthomonas campestris* pv. *glycines* and *Bacillus subtilis* were obtained from Bacteriology Laboratory, Research Center for Agriculture Biotechnology and Genetic Resources (BALITBIOGEN), Bogor, Indonesia and Laboratory of Industrial Microbiology, ENSAIA, Nancy, French respectively.

Mycelial Production. Mycelia were obtained from liquid cultures of *L. cladopus* LC4. One inoculant of 7 mm diameter mycelium of this isolate was inoculated on the surface of 100 ml of EMP media (malt extract 15 g, bacteriological peptone 5 g, glucose 20 g, distilled water 1 l) in 250 ml of Erlenmeyer flask. All cultures were incubated at 35 °C at static condition for 30 days.

Mycelial Extraction. Mycelia of the cultures were separated from the culture filtrate using filter paper. Mycelia were ground with a mortar and then extracted twice with 50 ml methanol and agitated overnight on a rotary shaker for each extraction. The extracts were separated from the mycelia with fritted glass filter no. 3 and then dried under vacuum on a 30 °C water bath and a rotary evaporator, then they were redissolved in methanol.

Activity Test of Extracts. The method used for extract activity test was paper disc method using *Xanthomonas campestris* pv. *glycines* as target microbe (Sudirman *et al.* 1994). The test media for this bacterium was Luria Bertani Agar (LBA) (tryptone 5 g, yeast extract 2.5 g, NaCl 5 g, agar 7.5 g, distilled water 1 l).

Cell suspensions of *X. campestris* pv. *glycines* obtained by inoculating one loop inoculator of bacterial colony to 20 ml Luria Bertani Broth. The cultures were agitated on a shaker at room temperature for two days. As much as 100 µl of the cell suspension were added into 100 ml of LBA media. Then the 10 ml of the media were poured on a Petri dish.

The extracts were prepared by placing 100 µl on a 13 mm paper disc. The treatment control was the discs with similar solvents without the extracts. The discs were dried on air in order to remove the solvents, sterilized with UV ray (254 nm) for 30 min and then placed on the surface of LBA media containing *X. campestris* pv. *glycines*. The media were then incubated at 10 °C for 3-4 hours in order to diffuse the extracts into agar media. After incubation at room temperature (28-30 °C) for two days, inhibition activities of extracts were estimated by measuring the diameters of inhibition zones.

Partial Purification with Column Chromatography. Three active compounds [R_f values of 0.83 (LC4-3 fraction), 0.73 (LC4-2 fraction), and 0.60 (LC4-1 fraction)] were purified by silicic acid column chromatography. The crude

extract were applied to a column (2.1 x 65 cm) filled with silica gel silicic acid (Merck 60, Nr. 9385) mixed with celite (BDH 15284) (1:1, w:w) and eluted with steps of increasing concentrations of methanol in dichloromethane: 0, 5, 10, 20, 60, 100%. The fractions were tested against *X. campestris* pv. *glycines* by paper disc method, detected by bioautography against *B. subtilis* and by being viewed under ultraviolet radiation at 366 nm (Sudirman 2005).

Detection of Extract Separation by Bioautography Method using *Bacillus subtilis*. The media for *B. subtilis* was Tryptone Glucose Yeast (TGY) (tryptone 5 g, yeast extract 5 g, glucose 1 g, K_2HPO_4 1 g, agar 7.5 g, distilled water 1 l). The cell suspensions of *B. subtilis* were obtained by growing 100 µl of stock suspension on 25 ml of TGY media in Petri dish and incubated at 28 °C for 15 days. Each Petri dish was then added with 3 ml of sterile distilled water, the suspension was then scrapped with glass rod, filtered with sterile cotton and pausterized three times at 60 °C for 30 min. One hundred microliter of this cell suspension were added to 100 ml of TGY media and then the media were poured on TLC plate explained below.

Thin-layer chromatography (TLC) analysis was carried out using silica gel plates (Merck 60 F 254, 0.1-mm thick, 20 x 5 cm). All fractions from column chromatography were deposited as spots and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Dry chromatograms were covered with 15 ml TGY medium containing *B. subtilis* and then incubated at 10 °C for 3-4 hours. The location of inhibition zones were estimated by measuring the R_f values of inhibition spots (Sudirman *et al.* 1994).

Partial Purification with Preparative thin Layer Chromatography. Three active compounds [R_f values were 0.83 (LC4-3 fraction), 0.73 (LC4-2 fraction), and 0.60 (LC4-1 fraction)] were further purified as well by preparative thin layer chromatography by using larger and thicker silica gel plates (Merck, Kieselgel G Nr. 7731, 2 mm thick, 20 x 20 cm). Crude mycelial extracts were deposited as spots along plates and developed in a butanol-acetic acid-water mixture (3:1:1, vol:vol:vol). Based on LC4-3, LC4-2, and LC4-1 R_f s, silica gels containing each active fraction were removed, extracted with methanol, and then dried under vacuum in a 30 °C water bath and a rotary evaporator, then redissolved in methanol. Each fraction was tested against *X. campestris* pv. *glycines*, detected by bioautography against *B. subtilis* and by being viewed under ultraviolet radiation at 366 nm (Sudirman 2005).

RESULTS

Antimicrobial Activity of *Lentinus cladopus* LC4 Mycelial Extracts. On the basis of experimental evidence, it appears that mycelial extract of tropical *L. cladopus* LC4 was considered to be further investigated due to its strong antimicrobial activity against *X. campestris* pv. *glycines*, a pathogen of soybean bacterial pustule disease with inhibition zone diameter up to 39 mm.

Partial Purification with Column Chromatography. Three fractions separated by silicic acid column chromatography i.e. FI, FII, and FIII eluted with

dichloromethane containing 0, 5, and 10% methanol respectively were active to inhibit growth of *X. campestris* pv. *glycines*. And the highest inhibition activity was performed by FII fraction (eluted with 5% methanol) (Figure 1). These three fractions were eluted with small volumes of eluents compare with other fractions, and they had lower fraction weights and concentrations (Figure 2). Other fractions i.e. FIV, FV, and FVI eluted with dichloromethane containing 20, 60, and 100% methanol respectively were not active despite they had higher concentrations, especially the FIV fraction.

All fractions on the chromatogram plates were separated into several spots (Figure 3). F1 fraction performed one fluorescence of active inhibition spot at R_f value of 0.8. FII fraction performed two fluorescence of active inhibition spots at R_f values of 0.8 and 0.7. And FIII fraction performed one fluorescence of active inhibition spot at R_f value of 0.8. But other fractions i.e. FIV, FV, and FVI fractions did not perform any active inhibition spot.

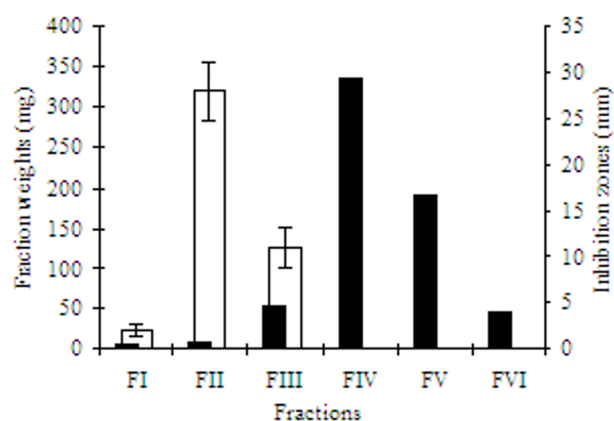


Figure 1. Partial purification of active compounds isolated from of *Lentinus cladopus* LC4 mycelia by silicic acid column chromatography eluted with dichloromethane containing methanol 0, 5, 10, 20, 60, and 100% and black bars of FI, FII, FIII, FIV, FV, and FVI indicated obtained fractions respectively. White bars indicated inhibition zone of the fractions against *Xanthomonas campestris* pv. *glycines*. (vertical bars indicated standar deviation, n = 5).

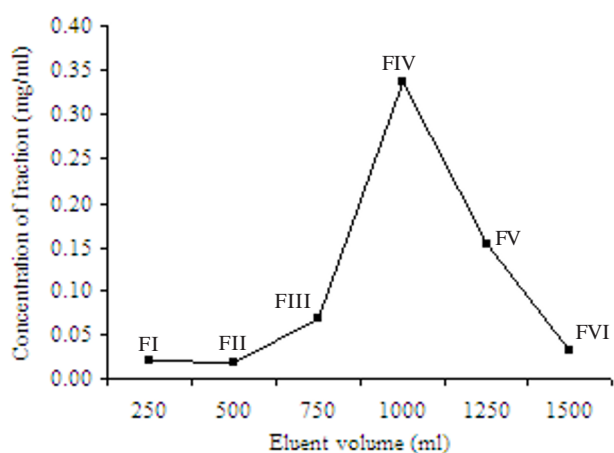


Figure 2. Elution profile of mycelial extract of *Lentinus cladopus* LC4 on silicic acid column chromatography eluted with dichloromethane containing methanol 0, 5, 10, 20, 60, and 100% and FI, FII, FIII, FIV, FV, and FVI indicated name of obtained fractions respectively.

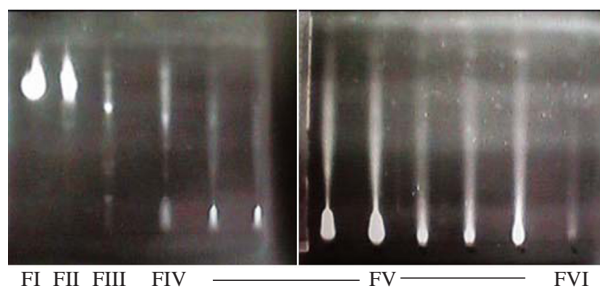


Figure 3. Partial purification of active compounds isolated from *Lentinus cladopus* LC4 mycelia by silicic acid column chromatography eluted by dichloromethane containing methanol 0% (FI), 5% (FII), 10% (FIII), 20% (FIV), 60% (FV), and 100% (FVI). The fractions were viewed under ultraviolet radiation at 366 nm, tested against *Xanthomonas campestris* pv. *glycines* (Xc) and detected by bioautography method using *Bacillus subtilis* for giving R_f values of inhibition zones.

Partial Purification with Preparative Thin Layer Chromatography. From three active compounds isolated from *L. cladopus* LC4 mycelia, LC4-2 fraction gave one inhibition zone with R_f value of 0.73. This fraction was fluorescence under ultraviolet radiation at 366 nm. LC4-3 fraction performed two inhibition zones with R_f values of 0.81 and 0.70 respectively. And LC4-1 fraction performed two inhibition zones with R_f values of 0.70 and 0.60 respectively. However, only LC4-2 fraction was active to inhibit growth of *X. campestris* pv. *glycines*.

DISCUSSION

The bioautographic results with *B. subtilis* showed that the crude extracts of tropical *Lentinus* spp. contained at least two active compounds. The previous results showed that *L. cladopus* LC4 produced eight active compounds isolated from fruiting body, mycelia, and culture filtrate extracts. Mycelial extract of *L. cladopus* LC4 contained three active compounds with R_f values of 0.83, 0.73, and 0.60 (Sudirman 2005). In this investigation, the compound with R_f 0.60 was not eluted from chromatographic column. This compound might be distributed in small quantity in each fraction of FV (Figure 3), as a consequence, the active compound can not be detected by bioautography method.

The separation of the active compounds isolated from *Lentinus cladopus* LC4 mycelial extract reduced the activity of each active compound against *X. campestris* pv. *glycines* as shown by FI and FIII fractions which each fraction contained one active compound with similar R_f values of 0.8. It was different from FII fraction which contained two active compounds with R_f values of 0.8 and 0.7 and it was more active against *X. campestris* pv. *glycines* than FI and FIII fractions. Different results with purification by preparative thin layer chromatography, only LC4-2 fraction containing one active compound with R_f value of 0.73 was active against this bacteria. Based on this result, it seems that mixture of compounds with R_f values of 0.8 and 0.7 will give stronger activity. Separation of these three antimicrobial compounds will be continued

to obtain the pure compounds for molecule characterization and will be focus on LC4-2 fraction.

All extracts of tropical *Lentinus* tested against *B. subtilis* always produce inhibition zones, except for the culture extract of *L. squarrosulus* LP9 (Sudirman 2009, unpublished data). *B. subtilis* was used for screening for bioautography instead of using *X. campestris* pv. *glycines* due to its sensitivity to antimicrobial compounds and its ability to grow on chromatogram plates.

The crude mycelial extract of *L. cladopus* LC4 had been tested *in vivo* against *X. campestris* pv. *glycines* on soybean leaves and seeds. The application of crude extract on the soybean leaves and on the healthy soybean seeds gave the highest inhibition of pustule formation as indicator of disease symptom, however it gave negative results for unhealthy seeds. Based on this result, the crude extract was supposedly to be used for preventive and curative application (Sudirman 2003, unpublished data). Recently the formula of biofungicide namely Bio-LC4 was formulated based on crude extract of *L. cladopus* LC4 and it has been patented. This formula was tested on the seedlings of flowering white cabbage (*Brassica rapa*). The results showed that the seedlings grown on the media containing the formula were less infected by disease than those grown without the formula (unpublished data). This research is still in progress in order to obtain the best formula for other pathogen targets.

It is suggested to screen edible mushrooms for anti-*Candida albicans* which could attack AIDS patients. Unfortunately, the fruiting body extracts of *L. torulosus* LU3 and *L. cladopus* LC6 were not active against this pathogen. Polysaccharide namely PSK from *Trametes versicolor* could inhibit the growth of *C. albicans* (Tsukagoshi *et al.* 1984). Other active compounds of polysaccharides such as lentinan extracted from shiitake mushroom (*Lentinula edodes*) and schizophyllan extracted from the split-gill mushroom (*Schizophyllum commune*) could inhibit growth of *C. albicans* and *Saccharomyces aureus* (Wasser & Weis 1999). Many non-edible mushrooms such as *Paecilomyces cicadidae* are potential in producing antimicrobial compounds as reported by Chen *et al.* (2008). Rosa *et al.* (2003) tested also the culture extract of *Phellinus* sp. against *Bacillus cereus*. Suay *et al.* (2000) found that all of the Ganodermataceae were specifically effective against *B. subtilis*. *G. applanatum* and also showed antimicrobial activity against *Staphylococcus aureus*, however they were less active against Gram-negative bacteria i.e. *E. coli*, and *Pseudomonas aeruginosa* (Smania *et al.* 2001).

Antimicrobial activity of the filtrate extract of *L. squarrosulus* 55A against *R. lignosus* F1 was reported, however the activity was due to the production of oxalic acid that was probably responsible for half of inhibitory effect of the filtrate extract. The active compound of *Lentinus* Ls2, had been characterized (Sudirman *et al.* 1994). Some properties of the Ls2 compound, such as the hydrophilic character; reactions with chromogenic reactants of sugars suggested that the molecule contains a glycosidic part; and the presence of alcoholic functions.

There might be possibility to find a strong active compound from other species of tropical *Lentinus* excluding *L. cladopus* LC4 which was being studied further. It is also interested to continue the research on *L. sajor-caju* LSC8 which produce two antimicrobial compounds from its mycelial extract (Sudirman 2005) as well as to study *L. squarrosulus* LP9. Both mushrooms are edible and active against enteropathogenic *Escherichia coli* (EPEC) which were resistant to several antibiotics (Sudirman 2009, unpublished data), so that they can be used either as food or medicine.

The purification of LC4 fraction (R_f 0.73) from crude extract will be further conducted using PTLC for better purity of the substances. And final purification of LC4-2 fraction to obtain better purity of the compound will be further conducted using PTLC and high performance liquid chromatography (HPLC). The pure compounds are necessary for characterization and determination of molecule structures. For this purposes, the pure extract will be characterized by chemical reactions, ultraviolet spectroscopy, infrared spectroscopy, proton nuclear magnetic resonance spectroscopy, and other physicochemical analysis.

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